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Examining the Cellular Pathways Involved in Influenza Virus Induced Apoptosis

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SUMMARY. Apoptosis is essential in many physiological processes including wound healing and development of the immune response. Apoptosis also plays an important role in the pathogenesis of many infectious diseases including those caused by viruses. Influenza viruses induce apoptosis in cells that are permissive for viral replication and cells that do not support viral replication. The cellular pathways involved in influenza virus induced apoptosis are currently ill defined. Previous studies suggest that influenza virus infection increased the expression of the Fas antigen in HeLa cells, and that Fas antigen is partially involved in apoptosis. In these studies we examined the cellular pathways involved in avian influenza virus induced apoptosis in two cell lines that support productive viral replication: Madin–Darby canine kidney cells (MDCK) and mink lung epithelial (Mv1Lu) cells.

RESUMEN. Examen de las rutas celeulares involucradas en la apoptosis inducida por el virus de influenza.

La apoptosis es esencial en muchos procesos fisiológicos incluyendo la cicatrización de heridas y el desarrollo de la repuesta inmune. La apoptosis juega igualmente un papel importante en la patogénesis de muchas enfermedades infecciosas incluyendo aquellas ocasionadas por virus. Los virus de influenza inducen apoptosis tanto en las células que permiten la replicación del virus como en las que no la permiten. Las rutas celulares involucradas en la apoptosis inducida por los virus de influenza no se encuentran aún bien definidas. Estudios anteriores sugieren que las infecciones por virus de influenza incrementan la expresión del antígeno Fas en células HeLa y que el antígeno Fas se encuentra involucrado parcialmente en la apoptosis. Se examinaron las rutas celulares involucradas en la inducción de apoptosis por el virus de influenza en dos líneas celulares que permiten la replicación productiva del virus; células MDCK y células epiteliales de pulmón de visón.

Key words: avian influenza, apoptosis, pathogenesis

Abbreviations: CEF = chicken embryo fibroblasts; FBS = fetal bovine serum; MDCK = Madin–Darby canine kidney; Mv1Lu = mink lung epithelial; SDS-PAGE = sodium dodecyl polyacrylamide gel electrophoresis; Tk/WI = A/Turkey/Wisconsin/68 H5N9; TTBS = Tris-buffered saline with 0.1% Tween-20; Ty/Ont = A/Turkey/Ontario/7732/67 H5N9

Virulent strains of influenza A viruses have caused severe losses to the poultry industry both in terms of mortality and cost of eradication (2,11,13,17,18). Many of the highly virulent strains exhibit dramatic destruction of lymphoid tissue (26,33,34) and endothelial cells (4,7,21) during infection. Until recently, the mechanism by which these highly virulent avian influenza (AI) strains cause the severe tissue damage and lymphocyte reduction was unknown. Previous studies demonstrated that all strains of influenza virus A induced programmed cell death or apoptosis in infected cells (8,10,12,20,22,23,25,27,28,30).

Apoptosis is essential in many physiological processes including tissue atrophy, development of the immune system, and tumor biology (3,15). Apoptosis also plays an important role in the pathogenesis of many infectious diseases, including those caused by viruses (19,24). Many virus infections result in apoptosis of host cells, and

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several viruses have evolved mechanisms to inhibit apoptosis (16,31). Although there is no obvious advantage for the induction of apoptosis by a cytopathogenic virus, influenza viruses induce apoptosis in numerous cell types both *in vivo* (14) and *in vitro* (10,22,23,35), and apoptosis could be an important antiviral host defense mechanism.

The cellular signals leading to apoptosis are varied and may depend on the viral strain and the cell type. Defining which viral proteins are toxic will be important for the understanding of pathogenesis. We showed that expression of the nonstructural 1 (NS1) protein of A/Turkey/Ontario/7732/67 H5N9 (Ty/Ont) and WSN results in apoptotic cell death through a mechanism involving the NS1 RNA-binding region (20). However, induction of apoptosis is not limited to the NS1 protein; the nucleoprotein (NP) and neuraminidase (NA) are also toxic to cells. These results demonstrate that numerous viral proteins cause cell death, perhaps using distinct cellular pathways. Therefore the aim is to define the cellular pathways involved in viral-induced apoptosis, determine which viral proteins are responsible, and ultimately understand the role of apoptosis in pathogenesis.

MATERIALS AND METHODS

Virus growth and cell culture. A/Turkey/Wisconsin/68 (Tk/WI) (H5N9, Southeast Poultry Research Laboratory Influenza virus repository) was propagated in the allantoic cavities of 10- or 11-day-old embryonated chicken eggs for 48 hr at 35°C. The allantoic fluid was harvested, centrifuged for clarification, and stored at −70°C. Madin–Darby canine kidney (MDCK) and mink lung epithelial (Mv1Lu) cells were grown in modified Eagle's medium (MEM, Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 2 mM glutamine. All cells were maintained at 37°C in 5% CO_2.

DNA fragmentation assay. Fragmentation of cellular DNA into the characteristic apoptotic ladder was assessed as previously described with minor modifications (22). Briefly, confluent monolayers infected with Tk/WI (multiplicity of infection of 10) or uninfected control cells were washed with phosphate-buffered saline (PBS) and incubated for varying time in tetracycline-free MEM containing 1% FBS. Prior to processing the DNA, cell monolayers were trypsinized and cell numbers determined. DNA was harvested by centrifuging the cells, resuspending the cell pellet in 300 µl of cold cell lysis buffer (10 mM Tris, 0.5% Triton X-100, pH 7.5), and incubating on ice for 30 min. The lysates were centrifuged at 12,500 rpm for 10 min at 4°C, and the supernatants were extracted once with buffered phenol and once with chloroform. The DNA was precipitated with 300 mM NaCl and ethanol. DNA samples were resuspended in 15 µl Tris-ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris, 1 mM EDTA, pH 7.5) treated with RNase A (Life Technologies). Equal cell numbers were loaded and electrophoresed through a 2% SeaKem GTG agarose gel (FMC Bioproducts, Rockland, ME). The gel was stained with ethidium bromide to visualize DNA fragmentation. Cell viability was also assessed by trypan blue exclusion.

Western blot analysis. Infected or control cell monolayers were lysed at 1, 3, 5, 8, and 24 hr postinfection, and total protein concentration was determined by Bradford colorimetric assay (BCA) assay (Pierce, Rockford, IL). Equal protein concentrations were heated for 3 min at 100°C in sample buffer containing β-mercaptoethanol and resolved by sodium dodecyl sulfate 5%–20% gradient polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to nitrocellulose, the proteins were blocked with 1% powdered milk in Tris-buffered saline with 0.1% Tween-20 (TTBS; Sigma Immunochemicals Inc., St. Louis, MO) for 30 min at room temperature. The nitrocellulose was probed for cellular proteins involved in apoptosis-specific mouse monoclonal or rabbit polyclonal antibodies diluted as per manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ) in TTBS and incubated for 1 hr at room temperature. Proteins were detected by incubation with a secondary antibody conjugated to horseradish peroxidase followed by enhanced chemiluminescence (Amersham, Arlington Heights, IL) following the manufacturer's protocols.

RESULTS

MDCK and Mv1Lu cell monolayers infected with the mildly pathogenic influenza strain Tk/WI are completely destroyed within 24 hr postinfection. DNA fragmentation, a measurement of apoptosis, is observed within 8 hr postinfection and increases throughout the 24 hr. Control cells show no DNA fragmentation. These studies demonstrate that a mildly pathogenic avian strain causes apoptosis similar to a highly pathogenic virus. The induction of apoptosis is not limited to mammalian cell lines; primary chicken embryo fibroblast cultures also undergo viral-induced apoptosis when infected with Tk/WI or Ty/Ont.

Unlike HeLa cells, Tk/WI infection of MDCK and Mv1Lu cells does not result in increased Fas or Fas ligand levels as measured by Western blot analysis or flow cytometry. However, there was a rapid increase in the levels of the tumor suppressor protein p53 within 5 hr postinfection. This increase

in p53 protein is prior to the appearance of DNA fragmentation, suggesting that p53 may be involved in viral-induced apoptosis.

To address the role of p53 in viral-induced apoptosis, we used two approaches: 1) a chemical inhibitor of p53 and 2) antisense oligodeoxynucleotides against p53. We found that inhibition of p53 results in decreased apoptosis, suggesting that p53 is involved in apoptosis.

Finally, studies are under way to determine the mechanism of increased p53 protein and measure p53 levels in chickens infected with mildly pathogenic or highly pathogenic AI to determine relevance *in vivo.*

DISCUSSION

Cells infected with influenza virus die whether by apoptosis or necrosis. This tissue damage plays a role in viral pathogenesis. What happens if we block the cell death? Would it be beneficial for the host or beneficial for the virus? Is tissue damage required for a proper host response? These are a few of the many questions we can address by understanding the mechanism of influenza virus induced cell death. Only in the last 10 years more research has focused on understanding viral induced apoptosis. Through these studies, we have gained increasing knowledge on the cell biology of viral infection. Unfortunately, most of the studies have focused on HeLa cells. The benefits of using HeLa cells are obvious; unlimited reagents are available to study apoptosis in human and mouse cells. HeLa cells do not support viral replication and originated from a human tumor, suggesting the cellular pathways in these cells may not be representative of a cell type that supports productive viral replication. Thus, we choose to focus determining the cell pathways in chicken embryo fibroblasts (CEFs), MDCKs, and Mv1Lu cells.

Our preliminary studies demonstrate that unlike HeLa cells (9,29,35), MDCK and Mv1Lu cells do not use a Fas and Fas ligand mediated cell death pathway. Instead the tumor suppressor protein, p53, is rapidly increased in viral infected cells. p53 is a DNA damage and response protein that is rapidly elevated in times of cellular stress (1,5,6,32,36). Because viral infection results in shutdown of host cell function and potentially in DNA damage, it is not surprising that p53 levels increase. More research is required to determine the exact role of p53 in viral-induced apoptosis.

Very few reagents are available to examine cellular pathways in avian cells. Initially these studies must be performed in mammals and mammalian cells. Future research will examine relevant cellular proteins in infected chickens and chicken cells, especially lymphoid cells. This will further our understanding of the host response against influenza virus infection at a cellular level.

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